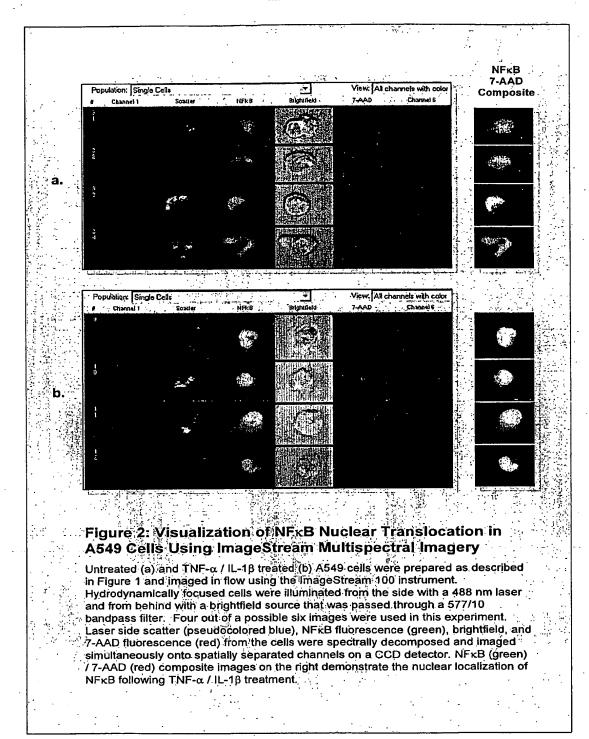


Fig. 1

bandpass filters appropriate for FITC (535/40 nm) and 7-AAD (630/60 nm) fluorescence. NFkB images in grey are depicted on the left. NFkB (green) / 7-AAD (red) composite images on the right demonstrate the nuclear localization.

of NFκB following TNF-α / IL-1β treatment.



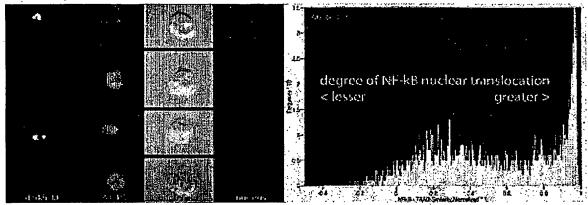


Figure 2C. NF-kB Nuclear Translocation In Immune Cells

The data (above left) show cells imaged simultaneously in darkfield, green fluorescence, brightfield, and red fluorescence. The sample consisted of a monocytic cell line stained with an antibody against the NF-kB transcription factor (green) as well as a nuclear stain (red). Cells treated with lipo-polysaccharide (image rows 2-4) exhibit translocation of NF-kB from the cytoplasm to the nucleus while untreated cells lack NF-kB in the nuclear compartment (top row). A statistical analysis of imagery from 6616 cells quantitatively characterizes the degree of NF-kB nuclear translocation in the sample. Amnis' ImageStream platform is the only cell analysis technology that can perform this valuable assay on immune cells in suspension.

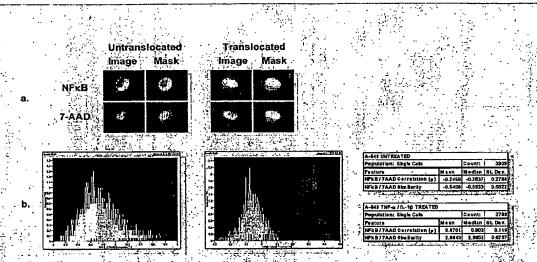


Figure 3: Quantitation of NFkB Nuclear Translocation Using Image Correlation Analysis

In order to quantitate the extent of NFkB nuclear localization, we analyzed the degree of pixel intensity correlation between the NFkB and nuclear images of each cell within a masked region of interest. The NFkB image of a cell with a high degree of translocation will took qualitatively similar to the nuclear image of that cell, resulting in a high degree of correlation between the two images while the NFkB image of cell without any translocation with have little signal in the nuclear space, resulting in an inverse correlation with the nuclear image. (A) shows the masked areas (turquoise overlay) used for the correlation analysis on an untranslocated and a translocated cell. Two features the correlation coefficient (p) and a logarithmic transformation of p (similarity) were calculated, and are represented by the following formulas:

$$\rho = \frac{Gov(X, F)}{\sigma_X \sigma_I}$$
Similarity = $\ln \left(\frac{1 + \rho}{1 - \rho} \right)$

p measures the degree to which the spatial distribution of intensities over two separate images is correlated, with a range from -1 (inverse correlation) to +1 (complete correlation): The similarity value ranges from -a to to, allowing standard statistical comparisons (means and standard deviations) between groups to be made. Histogram overlays of NFxB / 7-AAD correlation (b) and similarity (c) distinguish untreated (green) from TNF-a / IL-16 treated (red) A549 cells.

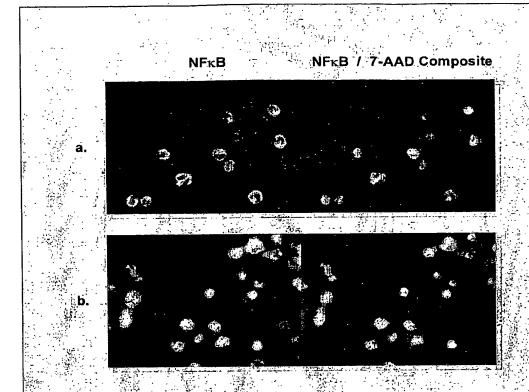


Figure 4: Visualization of NFkB Nuclear Translocation in THP-1.
Cells Using Immunofluorescence Microscopy

LPS stimulation initiates a signaling cascade that results in the translocation of NFκB from the cytoplasm to the nucleus of the non-adherent human monocyte cell line THP-1. Untreated THP-1 cells (a) and THP-1 cells treated with LPS (100 ng/ml) for 1 hour (b) were probed for NFκB expression and nuclear morphology. Briefly, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% triton, and incubated with mouse anti-NFκB (p65) + Alexa Fluor® 488 donkey anti-mouse IgG. Cells were washed and resuspended in 1% paraformaldehyde containing 7-AAD, then mixed with an equal volume of antifade and visualized on slides using a Nikon Eclipse E600 fluorescence microscope equipped with bandpass filters appropriate for FITC (535/40 nm) and 7-AAD (630/60 nm) fluorescence. Note the relatively thin band of cytoplasm in the untranslocated NFκB images characteristic of this monocytic cell line.

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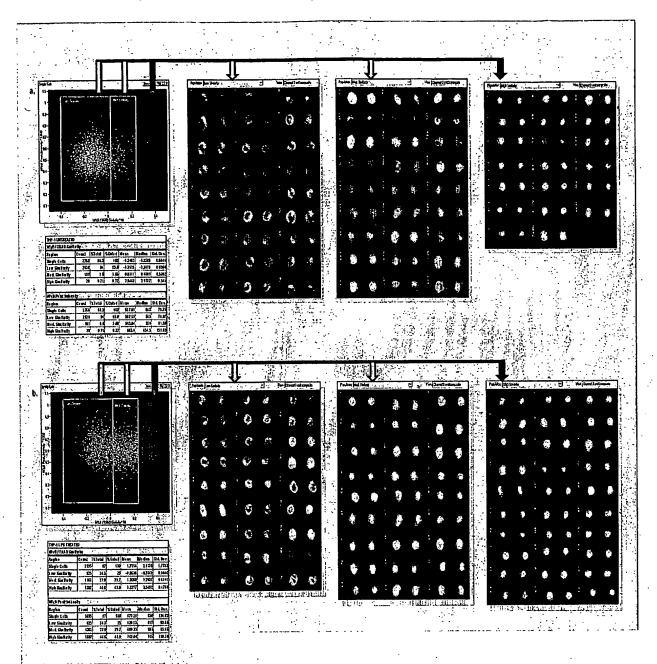


Fig. 5

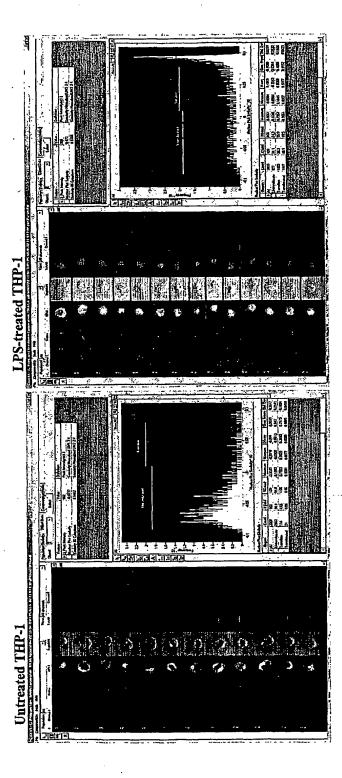


Fig. 6

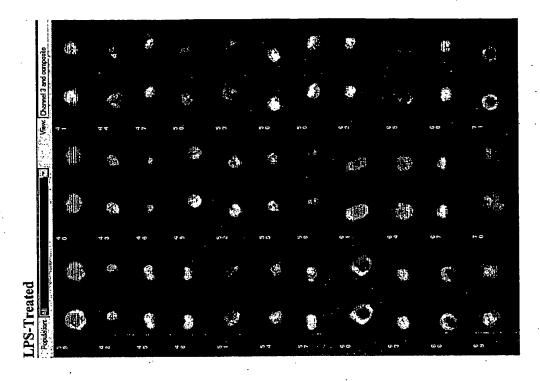
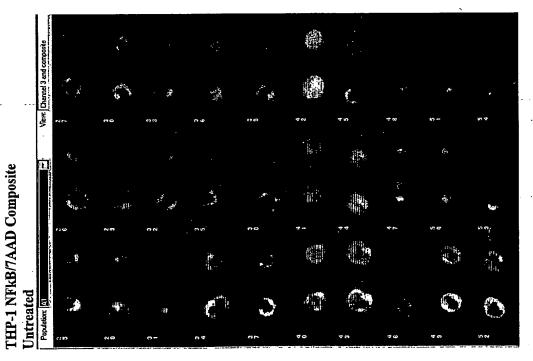


Fig. 7



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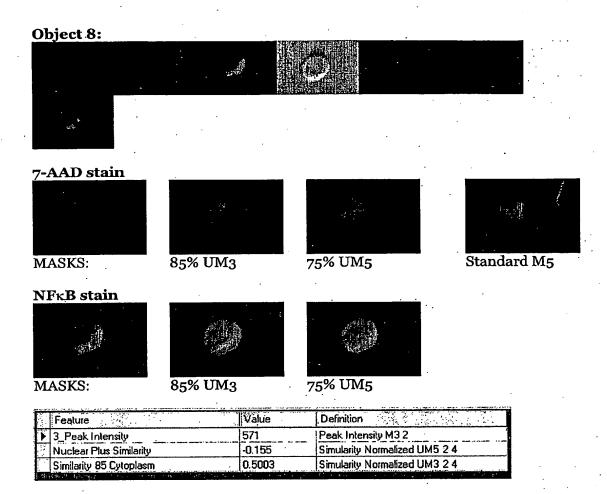
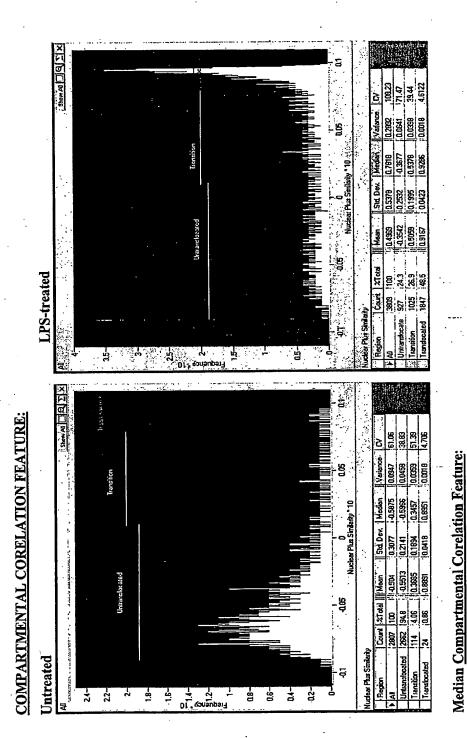


Fig. 8

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Translocated = 0.9286 + /- 0.0423

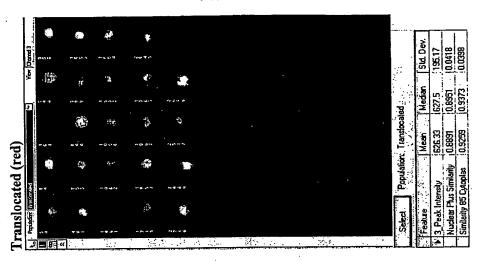
Untranslocated = -0.5966 +/- 0.2141

Difference of 1.5252

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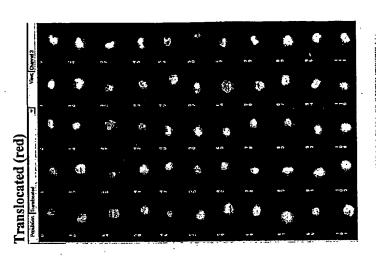


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Fig. 10A

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	Seleci	Population	Population: Translocated] B	
	Feature	7. A.M.	Mean	Median	Std Dev.
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	Similarity 85 Cytoplas	Cytoplas	0.946	0.9547	00307

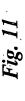
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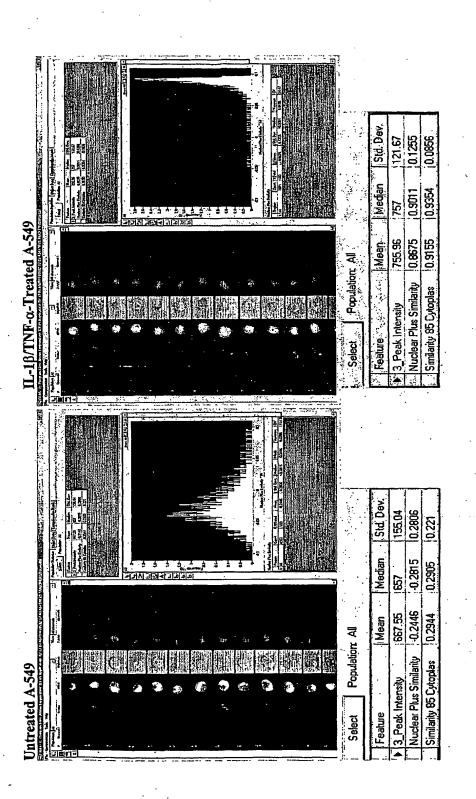
4 : E	Select " Population: T	r. Irensidon	* : ·	
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	Similarity 85 Cytoples	0.7633	0.7827	0.0829

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	Std Dev.	. 92.23	0.2532	a.1805	
ocated	Median	, 113;	-0.3677	0.4393	
Population: Untranslocated	Mean	52.23	-0.3542	10.4128	
Select Population	Feature	3_Peak Intensity	Nuclear Plus Similarity	Similarity 85 Cytoplas	

Fig. 10B





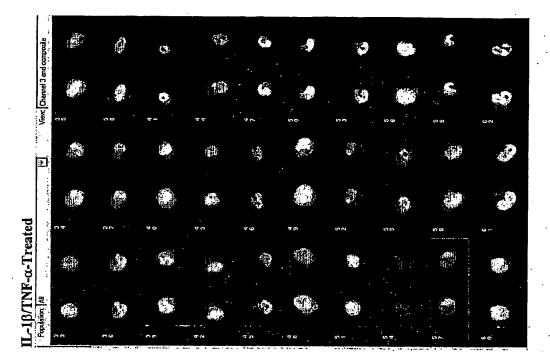
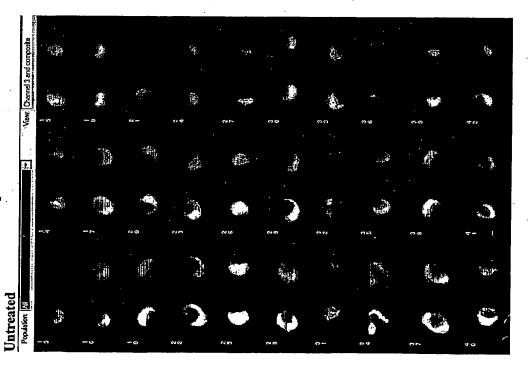
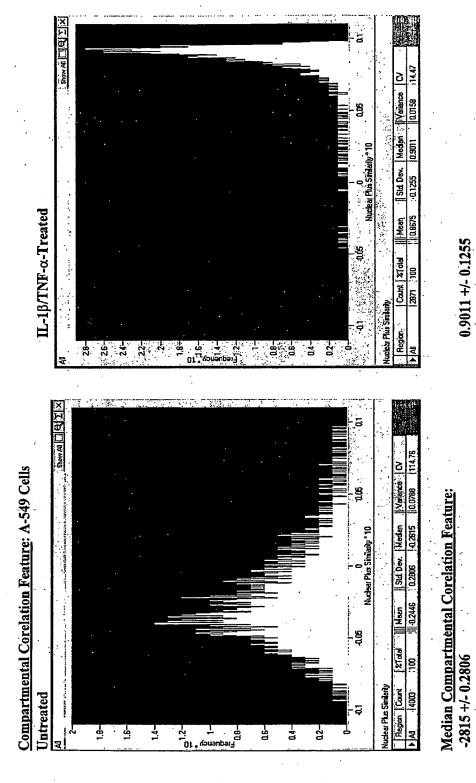


Fig. 12



A-549 NF-kB/7AAD Composite



0.9011 +/- 0.1255

Difference of 1.1826